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⑪ Publication number:

0 383 751 B1

⑫

EUROPEAN PATENT SPECIFICATION

⑯ Date of publication of patent specification: 09.03.94 ⑯ Int. Cl. 5: C12N 15/81, C12N 15/16,

⑯ Application number: 87906915.1 C12N 1/18, C12P 21/02,
C12N 1/20, C12R 1/19

⑯ Date of filing: 07.10.87

⑯ International application number:
PCT/EP87/00581

⑯ International publication number:
WO 88/03165 (05.05.88 88/10)

The file contains technical information submitted
after the application was filed and not included in
this specification

⑯ PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICROORGANISMS.

⑯ Priority: 22.10.86 US 921684

⑯ Date of publication of application:
29.08.90 Bulletin 90/35

⑯ Publication of the grant of the patent:
09.03.94 Bulletin 94/10

⑯ Designated Contracting States:
BE CH DE FR GB IT LI LU NL SE

⑯ References cited:
WO-A-84/01173
US-A- 4 546 082

⑯ Proprietor: SELMER SANDE A.S.
Josefines Gate 18
P.O. Box 7060
Homansbyen
N-0306 Oslo 3(NO)

Proprietor: ALESTROM, Peter
Bregnevn 21A
N-0875 Oslo(NO)

⑯ Inventor: GAUTVIK, Kaare, M.
Skovvn. 17
N-0257 Oslo 2(NO)
Inventor: BEATE OYEN, Tordis
Stordamvn. 33
N-0671 Oslo 6(NO)
Inventor: GABRIELSEN, Odd, Stokke
Ullernvn. 16
N-0280 Oslo 2(NO)

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Chemical abstracts, volume 102, n 7, 18 February 1985, (Columbus, Ohio, US), H.M. Kronenberg et al "Studies of parathyroid hormone secretion using recombinant DNA technology", see page 109, abstract 56590, Int. Congr. Ser. Excerpta Med. 1984, 619 (Endocr. Control Bone Calcium Metabol. vol. 8A), 217-20 (Eng)

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**Invent. r: ALESTROM, Peter
Bregnevn 21A
N-0875 Oslo(NO)**

**(2) Representative: Dost, Wolfgang,
Dr.rer.nat.,Dipl.-Chem. et al
Patent- und Rechtsanwälte,
Bardehle . Pagenberg . Dost . Altenburg .
Frohwitter . Geissler & Partner,
Postfach 86 06 20
D-81633 München (DE)**

DescriptionFIELD OF THE INVENTION

6 This invention relates to genetically engineered microorganisms containing DNA coding for human preproparathyroid hormone.

BACKGROUND OF THE INVENTION

10 A number of proteins and peptides that are normally synthesized by mammalian cells have proven to have medical, agricultural and industrial utility. These proteins and peptides may be of different molecular size and have a number of different functions, for example, they may be enzymes, structural proteins, growth factors and hormones. In essence both proteins and peptides are composed of linear sequences of amino acids which form secondary and tertiary structures that are necessary to convey the biological 15 activity. Human parathyroid hormone has a relatively small molecular weight, which has made it possible to synthesize the peptide chemically by the sequential addition of amino acids. Thus, parathyroid hormone is commercially available, but in very small quantities at high cost. As a result, there is no human parathyroid hormone available at a reasonable price to supply the many potential medical, agricultural and industrial applications.

20 During the past ten years, microbiological techniques employing recombinant DNA have made it possible to use microorganisms for the production of species-different peptides. The microorganism is capable of rapid and abundant growth and can be made to synthesize the foreign product in the same manner as bacterial peptides. The utility and potential of this molecular biological approach has already been proven by microbiological production of a number of human proteins that are now available for 25 medical and other uses.

Parathyroid hormone (PTH) is one of the most important regulators of calcium metabolism in mammals and is also related to several diseases in humans and animals, e.g. milk fever, acute hypocalcemia and otherwise pathologically altered blood calcium levels. This hormone therefore will be important as a part of diagnostic kits and will also have potential as a therapeutic in human and veterinary medicine.

30 The first synthesis of DNA for human preproparathyroid hormone was described by Hendy, G.N., Kronenberg, H.M., Potts, Jr. J.T. and Rich, A., 78 Proc. Natl. Acad. Sci. 7385-7389 (1981). DNA complementary in sequence to PTH mRNA was synthesized and made double stranded (Hendy et al., *supra*). This cDNA was cloned in pBR 322 DNA and *E. coli* 1776 was transfected. Of the colonies with correct antibiotic resistance, 23 out of 200 clones were identified as containing specific human PTH cDNA 35 inserts. However, none of the 23 human PTH clones contained the full length insert (Hendy et al., *supra*). Later, Born et al. (*Experientia* 39, 659, 1983) constructed vectors for expression of the cDNA of human prepro-PTH in bacteria. Whereas correctly processed HPTH could be detected upon disruption of the cells, soluble HPTH could not be found in the periplasmic space. Breyel, E., Morelle, G., Auf'mkolk, B., Frank, R., Blocker, H. and Mayer, H., Third European Congress on Biotechnology, 10-14 September 1984, Vol. 3, 363-40 described the presence of the human PTH gene in a fetal liver genomic DNA library constructed in the phage Charon 4A. A restriction enzyme fragment of the PTH gene was recloned and transfected into *E. coli*.

40 However, the work of Breyel et al., *supra*, demonstrated that *E. coli* degrades human PTH. Thus, a microorganism which shows a stable production of intact human parathyroid hormone has so far not been described. Further, secretion of parathyroid hormone has never before been obtained with yeasts, even 45 though WO 84/01173 describes a method for the production of mature proteins such as HPTH in yeasts. However, the few HPTH molecules, which were secreted, did not exhibit biological activity to a significant extent.

SUMMARY OF THE INVENTION

50 Accordingly, it is an object of the present invention to provide a plasmid for insertion in yeast containing DNA coding for parathyroid hormone. It is also an object of the present invention to provide a transformed yeast containing DNA coding for parathyroid hormone, including human parathyroid hormone (HPTH), and from which transformed yeast, parathyroid hormone may be obtained.

55 Other objects and advantages of the present invention will become apparent as the description thereof proc eds.

In satisfaction f th foregoing objects and advantages, there is provided by the present inv ntion a n v l plasmid for insertion in yeast, containing DNA coding for human preproparathyroid h rm n . The

plasmid for insertion into yeast of the present invention is distinguishable over prior art plasmids, for example as described in Hendy et al., *supra*, in that the plasmid of the present invention contains a double start codon at the 5' end of the DNA coding for preproparathyroid hormone. The presence of the double start codon may cause a production microorganism transformed with a plasmid containing this cDNA to produce preproparathyroid hormone at an increased rate and in an improved yield over prior art transformed microorganisms.

There is provided by the present invention a plasmid for insertion in yeast containing DNA coding for parathyroid hormone. In a preferred embodiment, this plasmid is prepared by recloning the plasmid for insertion in *E. coli* described above. Finally, the invention provides a yeast transformed by said plasmid for insertion in yeast such that the yeast produces and secretes parathyroid hormone. Thus, the invention provides a method by which parathyroid hormone may be isolated from yeast culture medium. In a preferred embodiment, the transformed yeast is *Saccharomyces cerevisiae*. In another preferred embodiment, the parathyroid hormone is human parathyroid hormone.

Samples of pSSHPTH-10, *E. coli* transformed therewith, pSS α LX5-HPTH1 and *Saccharomyces cerevisiae* transformed therewith were deposited in the American Type Culture Collection in Rockville, Maryland on September 29, 1986, under the provisions of the Budapest Treaty. The samples have been accorded the following deposit numbers:

Transformed *E. coli* containing pSSHPTH-10: ATCC 67223.

pSSHPTH-10: ATCC 40267.

Transformed *S. cerevisiae* containing

pSS α LX5-HPTH1: ATCC 20821.

pSS α LX5-HPTH1: ATCC 40268.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows all possible variations of the DNA sequence coding for human preproparathyroid hormone.

Figure 2 shows the specific human preproparathyroid hormone DNA coding sequence of the clone pSSHPTH-10.

Figure 3 shows a DNA Sequence coding for human preproparathyroid hormone and having a double start codon at the 5' terminal end with flanking sequences in which are shown all possible variations of the DNA which may be present on the plasmid of the present invention.

Figure 4 shows the specific human preproparathyroid hormone DNA coding sequence of the clone pSSHPTH-10 with flanking sequences.

Figure 5 shows the actual amino acids sequence of the human preproparathyroid hormone for which the DNA sequence in clone pSSHPTH-10 codes.

Figure 6 shows the composition of the recombinant plasmid pSSHPTH-10.

Figure 7 shows a map of pALX4.

Figure 8 shows the construction of pALX5 from pL4 and pMF α 1-1.

Figure 9 shows the construction and schematic drawing of pSS α LX5-HPTH1.

Figure 10 shows the sequence of the MF α 1-HPTH fusion gene with all possible combinations of the DNA coding for HPTH.

Figure 11 shows the sequence of the MF α 1-HPTH fusion gene.

Figure 12 shows an electrophoresis plate showing the human parathyroid hormone produced and secreted by yeast and recovered from the yeast culture medium.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As indicated above, the present invention is directed to a plasmid for insertion into yeast which contains DNA coding for parathyroid hormone and which is derived from the plasmid pSSHPTH-10 shown in Fig. 6. Finally, the invention is directed to a transformed yeast from which parathyroid hormone may be recovered.

The invention further provides methods of producing and isolating the plasmids and transformed microorganisms. Poly(A) selected RNA was isolated from human parathyroid adenomas collected immediately after surgery. The poly(A) RNA was enriched for correct size mRNA by ultracentrifugation through sucrose gradients. Preproparathyroid hormone of correct molecular weight was translated *in vitro* from this size fractionated poly(A) RNA as judged by sodium dodecylsulphate polyacrylamide gel electrophoresis after immun precipitation with antiparathyroid antiserum. The specific messenger RNA for the human PTH was used as template for complementary DNA synthesis using oligo d(T)18 as a primer and avian

myoblastosis virus reverse transcriptase. After removal of the RNA templates by alkali hydrolysis, the second strand complementary DNA was synthesized by incubating the purified first strand DNA in the presence of the Klenow fragment of *E. coli* DNA polymerase I. The double stranded complementary DNA was made blunt ended by the action of *Aspergillus oryzae* single strand specific endonuclease S1 and complementary DNA longer than 500 base pairs was isolated after neutral sucrose gradient centrifugation. Approximately 20 bases long d(C)-tail protrusions were enzymatically added to the 3' ends of the cDNA. This modified complementary DNA was annealed to restriction endonuclease *Pst*I cleaved and G-tailed vector pBR 322. Resulting recombinant plasmid DNA's were transformed into *E. coli* K12 BJ 5183. Positive transformants were analysed for by colony hybridization using two different synthetic deoxyribonucleotides which covered the N-terminal coding region as well as the 3' noncoding part of the hormone mRNA sequence, respectively. Six out of 66 clones that were positive for both probes were submitted for detailed analysis by restriction endonuclease mapping showing that they all were identical except for some size heterogeneity at the regions flanking the start codon and the *Xba*I site 3' for the stop codon. One clone, pSSHPTH-10, was subjected to DNA sequence analysis revealing a 432 nucleotide long human parathyroid hormone complementary DNA sequence inserted in the *Pst*I site of pBR 322. The entire cDNA sequence was found to be identical to the sequence previously described by Hendy, et al., *supra*, except for a five base pair deletion in front of the start codon.

Figure 2 shows the human preproparathyroid hormone DNA sequence of pSSHPTH-10. This may be compared with Figure 1, which shows all possible variations of the DNA sequence for human preproparathyroid hormone without the 5' double start codon. Figure 3 shows a DNA sequence coding for human preproparathyroid hormone and having a double start codon at the 5' terminal end with flanking sequences in which are shown all possible variations of the DNA which may be present on the plasmid of the present invention.

A plasmid coding for human preproparathyroid hormone is pSSHPTH-10, the DNA sequence of which, including the flanking sequence, is shown in Figure 4.

Figure 5 shows the DNA sequence coding for preproparathyroid hormone in pSSHPTH-10 with flanking sequences showing the corresponding amino acid sequence of preproparathyroid hormone.

The invention provides a plasmid for insertion into yeast containing DNA coding for parathyroid hormone.

Fig. 10 shows a partial DNA sequence for the plasmid for insertion into yeast in which: Nucleotide nos. 1-173 makeup the $MF\alpha 1$ promoter region and 5' noncoding sequence. 174-440 is the $MF\alpha 1$ N-terminal coding sequence. 441-695 is an HPTH sequence. 696-726 is an HPTH 3' noncoding sequence from pSSHPTH-10. 727-732 is from pUC19. 733-874 is $MF\alpha 1$ 3' noncoding sequence and transcriptional termination signal.

The parathyroid hormone may be human or animal parathyroid hormone, for example pig or bovine parathyroid hormone. The plasmid for insertion in yeast of the present invention may be recloned from plasmids containing DNA coding for human or animal parathyroid hormone.

Figure 11 shows the Nucleotide sequence of the $MF\alpha 1$ -HPTH fusion gene from pSS α LX5-HPTH1. Nucleotide nos. 1-173 makeup the $MF\alpha 1$ promoter region and 5' noncoding sequence. 174-440 is the $MF\alpha 1$ N-terminal coding sequence. 441-695 is the HPTH sequence obtained from pSSHPTH-10. 696-726 is an HPTH 3' noncoding sequence from pSSHPTH-10. 727-732 is from pUC19. 733-874 is $MF\alpha 1$ 3' noncoding sequence and transcriptional termination signal.

In a preferred embodiment, the plasmid for insertion in yeast contains DNA coding for human parathyroid hormone. As shown in the following examples, the HPTH sequence from pSSHPTH-10 has been recloned and inserted in specially designed vectors for expression in *Saccharomyces cerevisiae*.

pSSHPTH-10 was digested to form a 288 bp *Bgl*II-XbaI fragment. This fragment was then subcloned into pUC19 between the *Bam*HI and *Xba*I sites. The subclone was then digested with *Dpn* I, and the largest resulting fragment was isolated. The said fragment was then digested with *Sall*.

The plasmid pSS α LX5-HPTH1 that in yeast MAT α cells leads to the expression and secretion of PTH was constructed in three stages:

1. Construction of the yeast shuttle vector pL4 (which replicates in both *E. coli* and *Saccharomyces cerevisiae*).
2. Cloning of a DNA fragment containing the yeast mating pheromone $MF\alpha 1$ gene and its insertion into the yeast shuttle vector to make the p α LX5 vector.
3. Insertion of a DNA fragment from the coding region of the HPTH gene of pSSHPTH-10 into p α LX5 in reading frame with the prep part of the $MF\alpha 1$ gene, thereby producing the vector pSS α LX5-HPTH1.

The shuttle vector pL4 was constructed by inserting into pJD207, an EcoRI-Avall fragment containing the ADH1 promoter isolated from pADH040. A *Sph*I fragment was then deleted, resulting in a plasmid

pALX1. The *Pst*I Site in the β -lactamase gene was deleted and the plasmid was partially digested with *Pvu*I and *Bgl*II and ligated to a *Pvu*I *Bgl*II fragment of pUC8, to form pALX2. After a further oligonucleotide insert in, the plasmid was digested with *Hind*III and religated to form pALX4.

Total yeast DNA from the Y288C strain was digested with EcoRI, and the 1.6-1.8 kb fragments isolated.

5 These were ligated to EcoRI-cleaved pBR322, and *E. coli* was transformed. The clones were screened for MF α 1 inserts by oligonucleotide hybridization. The DNA selected thereby was then used to transform *E. coli*. The resulting plasmid pMF α 1-1 was digested with EcoRI, made blunt ended by Klenow enzyme, and then digested with *Bgl*III. The MF α 1 fragment was isolated, and ligated to pL5 (digested with *Bam*HI, made blunt ended with Klenow enzyme, and digested with *Bgl*III) to yield p α LX5.

10 In order to insert the human PTH cDNA fragment into p α LX5, the p α LX5 was digested with *Hind*III, creating sticky ends and the site was made blunt ended with the DNA polymerase I Klenow fragment and dNTP. The p α LX5 was then digested with *Sall* to create a sticky ended DNA complementary to the *Sall* digested human PTH fragment described above.

15 The *Sall* digested human PTH fragment was then inserted into the *Sall* digested p α LX5. The resulting plasmid pSS α LX5-PTH is shown in Figure 9.

pSS α LX5-PTH was then inserted into yeast, thereby transforming yeast so that the yeast produces and secretes human parathyroid hormone. In a preferred embodiment, the transformed yeast is *Saccharomyces cerevisiae*. An electrophoresis plate showing the human parathyroid hormone from the yeast culture medium is shown at Figure 12.

20 Although the method for making the plasmid for insertion in yeast by recloning pSSHPTH-10 is shown in detail, this method is shown to illustrate the invention, and the invention is not limited thereto. The method may be applied to a variety of other plasmids containing DNA coding for human or animal PTH to produce the plasmid for insertion in yeast of the present invention.

25 The plasmids of the present invention and transformed microorganisms were produced as set forth in the following examples.

EXAMPLE I

Isolation of mRNA and synthesis of complementary DNA (cDNA) of human parathyroid hormone.

30 Starting material for the invention was parathyroid adenomas obtained from patients by surgery. The parathyroid tissue was placed on dry ice directly after removal and transported to a laboratory for preparation of RNA. The frozen tissue was homogenized with an ultra Turax homogenizer in the presence of 4 M Guanidinium thiocyanate and the RNA content was recovered by serial ethanol precipitations as described by Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J., 18 Biochemistry 5294-5299 (1979). The RNA preparation was applied to oligo d(T) cellulose affinity chromatography column in order to enrich for poly(A) containing mRNA. The poly(A) rich RNA was further enriched for parathyroid hormone (PTH) mRNA sized RNA by ultracentrifugation through a 15-30% linear sucrose gradient. The resulting gradient was divided into 25 fractions and every third fraction was assayed for PTH mRNA content by in vitro translation followed by immunoprecipitation with anti PTH antiserum (Gautvik, K.M., Gautvik, V.T. and Halvorsen, J.F., Scand. J. Clin. Lab. Invest. 43, 553-564 (1983)) and SDS-polyacrylamide gel electrophoresis (Laemmeli, U.K., 227 Nature 680 (1970)). The RNA from the fractions containing translatable PTH mRNA was recovered by ethanol precipitation.

40 This RNA, enriched for PTH mRNA, was used as a template for cDNA synthesis using oligo d(T)18 as a primer and avian myoblastosis virus reverse transcriptase for catalysis of the reaction (Maniatis, T., Fritsch, E.F. and Sambrook, J., Molecular Cloning pp. 230-243 (1982)). After first strand synthesis, the RNA templates were removed by alkali hydrolysis. The second strand cDNA was synthesized by incubating the purified first strand cDNA in the presence of the Klenow fragment of *E. coli* DNA polymerase I (Maniatis, supra). This in vitro synthesized double stranded cDNA was made blunt ended by the action of *Aspergillus oryzae* single strand specific endonuclease S1 (Maniatis, supra). The blunt ended double stranded cDNA was size fractionated over a 15-30% neutral sucrose gradient. The size distribution of each fraction was estimated by agarose gel electrophoresis together with known DNA fragment markers. Fractions containing cDNA larger than approximately 500 base pairs were pooled and the cDNA content was collected by ethanol precipitation.

EXAMPLE 2**Cloning of cDNA PTH in plasmid pBR 322 and transformation of E. coli K12 BJ5183.**

5 An approximate 20 base long d(C)-tail protrusion was enzymatically added to the 3' ends of the cDNA by the action of terminal deoxynucleotidyl transferase (Maniatis, *supra*). The d(C)-tailed cDNA was annealed to restriction endonuclease PstI cleaved and d(G)-tailed vector pBR322 and the resulting recombinant plasmid DNA's were transformed into E. coli K12 BJ 5183 cells which were made competent by the method of Hanahan, D., 168 *J. Mol. Biol.* 557-580 (1983). A total of 33,000 transformants were analyzed for PTH

10 cDNA content by colony hybridization (Hanahan, D. and Meselson, 10 *Gene* 63 (1980)).

15 Two to three thousand transformants were plated directly on each 82 mm diameter nitrocellulose filter, placed on top of rich medium agar plates containing tetracycline, and incubated at 37 degrees Centigrade until approximately 0.1 mm diameter colonies appeared. Duplicate replicas of each filter was obtained by serial pressing of two new filters against the original filter. The replica filters were placed on top of new tetracycline containing agar plates and incubated at 37 degrees Centigrade until approximately 0.5 mm diameter colonies appeared. The master filter with bacterial colonies was kept at 4 degrees Centigrade placed on top of the agar plate and the duplicate replica filters were removed from the agar plates and submitted to the following colony hybridization procedure.

20 EXAMPLE 3**Characterization of bacterial clones containing recombinant PTH cDNA and of the DNA sequence of clone pSSHPTH-10.**

25 The cells in the respective colonies were disrupted *in situ* with alkali and sodium chloride leaving the DNA content of each bacterial clone exposed: The procedure allows the DNA to bind to the filter after which it was neutralized with Tris-buffer and dried at 80 degrees Centigrade. The majority of cell debris was removed by a 65 degree Centigrade wash with the detergent sodium dodecylsulphate (SDS) and sodium chloride leaving the DNA bound to the filters at the position of the former bacterial colonies. The filters were

30 presoaked in 6xSSC (0.9M NaCl, 0.09M Na-citrate), 1x Denhart's solution (0.1 g/ml Ficoll, 0.1 g/ml polyvinyl pyrrolidone, 0.1 g/ml bovine serum albumin),

100 μ g/ml herring sperm DNA, 0.5% SDS and 0.05% sodium pyrophosphate for two hours at 37 degrees Centigrade (Woods, D.E., 6 *Focus* No. 3. (1984)).

35 The hybridization was carried out at 42 degrees Centigrade for 18 hours in a hybridization solution (6x SSC, 1x Denhart's solution, 20 μ g/ml tRNA and 0.05% sodium pyrophosphate) supplemented with 32P-labelled DNA probe (Woods, *supra*).

40 The DNA used as a hybridization probe was one of two different synthetic deoxyribo oligonucleotides of which the sequences were deduced from the published human PTH cDNA sequence of Hendy, et al., *supra*. The first probe was a 24-mer oligonucleotide originating from the start codon region of the human preproPTH coding sequence having a nucleotide sequence reading TACTATGGACGTTTCTGTACCGA. The second oligonucleotide was a 24-mer spanning over a cleavage site for the restriction endonuclease XbaI located 31 nucleotides downstream of the termination codon and consisted of the nucleotide sequence CTCAAAGACGAGATCTGTCACATCC.

45 Labelling was carried out by transfer of 32 P from 32 P- γ -ATP to the 5' end of the oligonucleotides by the action of polynucleotide kinase (Maxam, A.M. and Gilbert, W., 65 *Methods Enzymol.*, 499 (1980)).

50 The hybridized filters were washed in 6xSSC, 0.05% sodium pyrophosphate at 42 degrees Centigrade prior to autoradiography. Sixty-six clones were found to be positive for both probes as judged from hybridization to both copies of the duplicate replica filters. All those were picked from the original filters with the stored cDNA library and amplified for indefinite storage at -70 degrees Centigrade. Six of these were chosen for plasmid preparation and a more detailed analysis by restriction endonuclease mapping, showing that all were identical except for some size heterogeneity at the regions flanking the start codon and XbaI site, respectively.

EXAMPLE 4

Clon pSSHPTH-10.

5 One clone, pSSHPTH-10, was subjected to DNA sequence analysis according to the method of Maxam and Gilbert, *supra*. The complete structure of pSSHPTH-10 is shown in Figure 6. This clone consists of a 432 base pair long PTH cDNA sequence inserted in the PstI site of pBR322 having 27 G/C base pairs at the 5' end and 17 G/C base pairs at the 3' end. The complete DNA sequence of the cDNA insert of pSSHPTH-10 is shown in Figure 4. It is identical to the sequence of Hendy, et al., *supra*, except for a five
10 base pair deletion right in front of the start codon, changing the published (Hendy, et al., *supra*) start-stop (ATGTGAAG) signal (deletion is underlined) preceding the used start codon (ATG) to a double start signal (ATGATG).

EXAMPLE 5

15 Construction of the yeast shuttle vector pL4.

Before the HPTH-yeast-expression project was initiated, a family of general yeast expression vectors were developed. One of these, pL4, later was used to make pSS_αLX5-HPTH1, as described below:

20 The plasmid pJDB207, constructed by Beggs, J.D., "Multiple-copy yeast plasmid vectors," Von Wettstein, D., Friis, J., Kielland-Brandt, M. and Stenderup, A. (Eds) *Molecular Genetics in Yeast* (1981), Alfred Benzon Symposium Vol. 16, 383-390, was chosen as the basis for the general expression vectors. It contains an EcoRI fragment of the yeast 2 micron DNA inserted into the pBR322 derivative pAT153. It also contains the yeast LEU2 gene. The copy number of pJDB207 in yeast *cir^r* cells is very high relative to that
25 of other plasmids and it is unusually stable after non-selective growth in a *cir^r* strain. Parent, S.A., Fenimore, C.M., and Bostian, K.A. "Vector Systems for the Expression, Analysis and Cloning of DNA Sequences in *S. cerevisiae*," 1 *Yeast* 83-138 (1985); Erhart, E. and Hollenberg, C.P., "The Presence of a Defective LEU2 Gene on 2 Micron DNA Recombinant Plasmids of *Saccharomyces cerevisiae* is Responsible for Curing and High Copy Number," 156 *J. Bacteriol.* 625-635 (1983). These properties are related to a
30 partial defective promoter in the selective marker gene LEU2 (often named LEU2d, d for defective), Erhart et al., *supra*, which is not changed in the following constructs.

A 1260 base pair EcoRI-Avall fragment containing the ADHI promoter was isolated from the plasmid pADH040. After a fill in reaction with the Klenow fragment of DNA polymerase I and all four dNTPs, BamHI linkers were attached and the fragment was cloned into the unique BamHI site of pJDB207. From the
35 plasmid with the promoter in a counterclockwise direction, a 1050 base pair SphI fragment was then deleted (from the SphI site in pJDB207 to the SphI site in the promoter fragment) leaving only a single BamHI site. This plasmid was designated pALX1.

The PstI site in the β -lactamase gene of pALX1 then was eliminated without inactivating the gene. pALX1 was digested to completion with PstI and nuclease S1 to destroy the PstI site, and then subjected to
40 a partial digestion with Pvul and BgII. At the same time, a 250 base pair Pvul BgII fragment was isolated from pUC8, Vieira, J. and Messing, J., 19 *Gene* 259 (1982), that contains the corresponding part of a β -lactamase gene without a PstI site. This was ligated to the partially digested pALX1. In all the ampicillin
45 resistant clones isolated the β -lactamase gene had been restored by incorporating the pUC8 fragment. This plasmid was called pALX2.

45 The following oligonucleotide was purchased from Prof. K. Kleppe, University of Bergen, and inserted into the BamHI site of pALX2:

50 BglII * ↓↓* * HindIII
 GATCAGATCTGCAGGATGGATCCAAAGCTT ↓↓: initiation codon
TCTAGACGTCCTACCTAGGTTCGAACTAG * : optimal ATG context
PstI BamHI

Plasmids with the proper orientation were isolated and designated pALX3.

55 Finally the pALX3 was digested with HindIII and religated to delete a HindIII fragment of 480 base pairs. The resulting vector is called pALX4. A map of pALX4 is shown in Figure 7.

pL4 is a derivative of pALX4 in which the ADHI promoter is deleted. pL4 was used as basis for the insertion of other promoters. pALX4 was first digested with BgIII and Sall. The resulting sticky ends were

filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, followed by religation. By this treatment, the ADH1 promoter is eliminated and the BgIII site regenerated to give the vector pL4.

EXAMPLE 6

5

Construction of α LX5.

The gene for the yeast mating pheromone MF α 1 was first cloned by Kurjan, J. and Herskowitz, I., "Structure of a Yeast Pheromone Gene (MF α): A Putative α -Factor Precursor Contains Four Tandem Copies of Mature α -Factor", 30 Cell, 933-943 (1982). The published sequence was used to reclone the MF α 1 gene. Total yeast DNA from the strain Y288C was digested with EcoRI and digestion products in the size range from 1.6 to 1.8 kb were isolated from a preparative agarose gel. These were then ligated to dephosphorylated EcoRI cleaved pBR322 and used to transform E. coli BJ5183. The resulting clones were screened for MF α 1 gene inserts by hybridization to a labeled oligonucleotide of the following composition:

15 TGGCATTGGCTGCAACTAAAGC

DNA from purified positive clones was then used to transform E. coli JA221 from which plasmid DNA was prepared. The plasmid used in the following constructs, pMF α 1-1, is shown in Figure 8.

pMF α 1-1 was digested with EcoRI, filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with BgIII. The 1.7 kb MF α 1 gene fragment was isolated from an 20 agarose gel. Before inserting it into the yeast shuttle vector, the HindIII site of pL4 was eliminated by HindIII digestion, Klenow fill-in reaction and religation to give the pL5 shuttle vector. pL5 was digested with BarnHI, filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with BgIII. After purification on gel it was ligated to the MF α 1 fragment to give the expression vector α LX5 as shown in Figure 8.

25

EXAMPLE 7

Construction of pSS α LX5-HPTH1

30 A 288 base pair BgIII XbaI fragment from the pSSHPTH-10 plasmid was isolated and subcloned in pUC19 using the BarnHI and XbaI site of this vector. This subclone designated pUC-HPTH, was digested with DpnI and the largest fragment isolated. This fragment was then digested with Sall and the smallest of the two resulting fragments was again isolated, yielding a sticky end on the Sall cut side and a blunt end at the DpnI cut side.

35 α LX5 was digested with HindIII, filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with Sall. After purification from gel, it was ligated to the HPTH fragment described above. The resulting clones had the HindIII site regenerated verifying that the reading frame was correct. This plasmid called pSS α LX5-HPTH1 is shown in Figure 9. The sequence of the MF α 1-HPTH fusion gene is shown in Figure 10.

40

EXAMPLE 8

Expression And Secretion Of HPTH In Yeast

45 The yeast strain FL200 (α , ura3, leu2) was transformed with the plasmids α LX5 and pSS α LX5-HPTH1 using the spheroplast method. One transformant of each kind was grown up in leu⁻ medium and aliquots of the cell-free medium were analysed by SDS-PAGE developed by silver-staining (Fig. 12). Two major bands were seen in the medium from the pSS α LX5-HPTH1 transformant that were not present in the medium from the α LX5 transformant: one band of approximately 9000 daltons, the expected size of HPTH, and one band of approximately 16000 daltons that could correspond to an unprocessed MF α 1-HPTH fusion product. Both polypeptides reacted with antibodies against human PTH in a manner identical to the native hormone.

50 The examples are included by way of illustration, but the invention is not limited thereto. While the above examples are directed to providing a *S. cerevisiae* which produces and excretes human parathyroid hormone, the method of the present invention may be applied to produce a plasmid containing DNA coding for parathyroid hormone from any species. Further, said plasmid may be inserted into any species of yeast. The invention thus is not limited to *S. cerevisiae*.

55 The cloned human parathyroid hormone produced by the yeast of the present invention has a variety of known and potential uses. For example, it is current medical theory that human parathyroid hormone will be

highly effective in treating osteoporosis. Genetically engineered parathyroid hormone may be useful in an analytical kit for measuring parathyroid hormone levels in humans and animals. Human parathyroid hormone or fragments thereof may also be used for treatment of humans or animals displaying reduced or pathologically altered blood calcium levels. It is anticipated that many other uses will be discovered when 6 genetically engineered parathyroid hormone is available in large quantities, for example as a result of the present invention.

The invention has been described herein with reference to certain preferred embodiments.

Claims

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1. A plasmid for insertion in yeast comprising a nucleotide sequence coding for production and secretion of parathyroid hormone, more particularly human parathyroid hormone wherein the nucleotide sequence comprises:

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10 30 50
 AGTCAAGAAAAACCAAAAGCAACAAACAGGTTGGATAAGTACATATATAAGAGGGCCT
 5 70 90 110
 TTGTTCCATCAAAATGTTACTGTTCTACGATTCACTACGATTCAAGAAATAGTC
 10 130 150 170
 AACAGAAGATTACAAACTATCAATTCTATACACAAATATAACGACCAAAAGAAATGAGAT
 15 190 210 230
 TTCCTTCAATTCTTACTGCAGTTTATTGCAGCATCCTCCGCATTAGCTGCTCCAGTC
 20 250 270 290
 ACACATACAAACAGAAGATGAAACGGCACAAATTCCGGCTGAGCTGTCATCGGTTACTCAG
 25 310 330 350
 ATTAGAAGGGGATTGATGTTGCTGTTGCCATTTCACAGCACAAATAACGGGT
 30 370 390 410
 TATTGTTATAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGTATCTTGG
 35 430 450 470
 ATAAAAGAGAGGCTGAAGCTWSNGTWSNGARATHCARYTNATGCAYAAAYTNGGNAARC
 40 490 510 530
 AYYTNAAYWSNATGGARMGNNGARTGGYTNMGNAARAARYTNCAKGAYGTNCAYAAAYT
 45 550 570 590
 TYGTNGCNYTNGGNGCNCCNYTNGCNCCNMNGAYGCNGGNWSNCARNGNCCNMGNAAARA
 50 610 630 650
 ARGARGAYAAYGTYTNGTNGARWSNCAYGARAARWSNYTNGGNGARGCNGAYAARGCNG
 55 670 690 710
 AYGTNAAYGTYTNAACNAARGCNAARWSNCARTRRAATGAAAACAGATATTGTCAGACT
 60 730 750 770
 TCTGCTCTAGAGTCGACTTTGTTCCACTGTACTTTAGCTCGTACAAATACAAATAC
 65 790 810 830
 TTTTCATTCTCCGTAACAAACCTGTTTCCATGTAATATCCCTTTCTATTTCGTT
 70 850 870
 CGTACCAACTTACACATACATTATATAAGCTAT,

wherein M = A or C; R = A or G; W = A or T; S = C or G; Y = C or T; H = A or C or T; N = A or G or C or T, nucleotides 1 to 173 of said sequence making up the MF α 1 promoter region and 5' non-coding sequence; nucleotides 174 to 440 being the MF α 1 N-terminal coding sequence; nucleotides 441 to 695 being an HPTH sequence, nucleotides 696 to 726 being an HPTH 3' non-coding sequence from pSSHPTH-10 shown in Figure 6, nucleotides 727 to 732 being from pUC 19, nucleotides 733 to 874 being the MF α 1 3' non-coding sequence and transcriptional termination signal.

2. The plasmid of claim 1 wherein the nucleotide sequence comprises:

10 10 30 50
AGTGCAAGAAAACCAAAAAGCAACAAACAGGTTTGGATAAGTACATATATAAGAGGGCCT

15 70 90 110
TTTGTCCCCATCAAAAAATGTTACTGTTCTTACGATTCAATTACGATTCAAGAAATAGTTCA

130 150 170
22 AACAAAGAAGATTACAAACTATCAATTTCATACACAATATAAAAGCACCAGAATGAGAT

190 210 230
TTCCCTTCAATTCTTACTGCAGTTTATTGCAGCATCCCTCCGATTAGCTGCTCCAGTC

250 270 290
ACACTACAAACAGAAGATGAAACGGCACAAATCCGGCTGAAGCTGTATCGGTTACTCAG

30 310 330 350
ATTTAGAAGGGGATTCGATGTTGCTTTGCCATTTCCAACAGCACAAATAACGGGT

35 370 390 410
TATTGTTTATAAATCTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGTATCTTTGG

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430 450 470
 · ATAAAAGAGAGGGCTGAAGCTTCTGTGAGTGAAATACAGCTTATGCATAACCTGGGAAAC

 490 510 530
 ATCTGAACTCGATGGAGAGAGTAGAAATGGCTGCGTAAGAAGCTGCAGGATGTGCACAATT

 550 570 590
 TTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTCCAGAGGCCCGAAAAA

 610 630 650
 AGGAAGACAATGTCTGGTTGAGAGCCATGAAAAAAAGTCTGGAGAGGCAGACAAAGCTG

 670 690 710
 ATGTGAATGTATTAACATAAGCTAAATCCCAGTAAAAATGAAAACAGATATTGTCAGAGT

 730 750 770
 TCTGCTCTAGAGTCGACTTTGTTCCCACTGTACTTTAGCTCGTACAAAATACAAATATAC

 790 810 830
 TTTTCATTTCTCCGTAAACAAACCTGTTTCCATGTAATATCCTTTCTATTTTCTGTT

 850 870
 CGTTACCAACTTACACACATACTTATATAGCTAT,

35 wherein nucleotides 1 to 173 of said sequence make up the MF α 1 promoter region and 5' non-coding sequence; nucleotides 174 to 440 are the MF α 1 N-terminal coding sequence; nucleotides 441 to 695 are an HPTH sequence, nucleotides 696 to 726 are an HPTH 3' non-coding sequence from pSSHPTH-10, shown in Figure 6, nucleotides 727 to 732 are from pUC 19, nucleotides 733 to 874 are the MF α 1 3' non-coding sequence and transcriptional termination signal.

40 3. A microorganism in the form of a yeast preferably *Saccharomyces cerevisiae*, containing the plasmid of
claim 1 or 2.

45 4. A method of making the plasmid of claim 1 or 2, comprising the insertion of the nucleotide sequence of
claim 1 or 2, coding for PTH, particularly human PTH, into a plasmid.

45 5. The method of claim 4, wherein the insertion is carried out by recloning pSSHPTH-10, the restriction
map of which is shown in Fig. 6.

Patentansprüche

50 1. Plasmid zur Insertion in Hefe, umfassend eine Nucleotidsequenz, die für die Herstellung und Absondierung von Parathormon und insbesondere für menschliches Parathormon codiert, wobei die Sequenz folgende Nucleotide umfaßt:

10 AGTGCAGAAGAAGAAGCCAGAGCAGAAGCAGGTTGGATAGTACATATATAAGAGGGCT
 20 TTTGTTCCATCAAGAAGTACTCTTCTTACGATTCAATTACGATTCAAGAAGTACTCA
 30 AACAGAAGAAGATTAACAACTATCAATTACAAATATAAACGACCCAAAGAATGAGAT
 40 TTCCCTCAAAATTTACTGCAGTTATTCGCAGCATTCTCCGCATTAGCTGCTCCAGTC
 50 ACACCTACAAACAGAAGATGAAACGGCACAAATCCGGCTGAGCTGTCACTCGTTACTCAG
 60 ATTAGAAAGGGGATTCCATTTGCTGTTGCCATTTCACAGCACAAATAAACGGGT
 70 TATTGTTATAAAACTACTATTGCCAGCATTCGCTAAAGAAAGAAGGGGTATCTTGC
 80 ATAAAGAGAGGGCTGAGCTWSNGINWSNGARATYCARYTNAATCCAYAAATYINGGNAR
 90 AYYTNAAYWSNAATGGARMGNGTNGARTGGYTNMGNAARAAARYTNCARGAYGTNCAYAAAT
 100 TYGTINGCNYTNGGGCNCCNYTNGCNCCNMNGAYGCNGCNWSNCARMGNCCNMGNAA
 110 ARGARGAYAAAGTNYTNGTNGARWSNCAYGARAARWSNYTGGNGARGCNGAYAARGCNG
 120 AYGTNAAYGTNYTNAAGCNAARGCNAARWSNCARTRRAATGAAAACAGATATGTCAAGAGT

730 750 770
TCTGCTCTAGAGTCGACT...TGTCCACTGTACT...TACCTCGTACAAAATACAAATAC

6 790 810 830
-----C-----C-----C-----A-----A-----C-----C-----G-----T-----A-----A-----T-----C-----T-----G-----C-----C-----C-----

10 850 870
CCTTACCAACTTACACATACCTTATATAGCTAT,

15 wobei M = A oder C; R = A oder G; W = A oder T; S = C oder G; Y = C oder T; H = A oder C oder T; N = A oder G oder C oder T, wobei die Nucleotide 1 bis 173 der Sequenz die MF α 1-Promotorregion und die 5'-nicht-codierende Sequenz ausmachen, es sich bei den Nucleotiden 174 bis 440 um die MF α 1-N-terminale codierende Sequenz handelt, es sich bei den Nucleotiden 441 bis 695 um eine HPTH-Sequenz handelt, es sich bei den Nucleotiden 696 bis 726 um eine HPTH-3'-nicht-codierende Sequenz aus pSSHPTH-10 handelt, das in Fig. 6 gezeigt ist, die Nucleotide 727 bis 732 aus pUC19 stammen und es sich bei den Nucleotiden 733 bis 874 um die MF α 1-3'-nicht-codierende Sequenz und das transkriptionale Terminationssignal handelt.
20

2. Plasmid nach Anspruch 1, wobei die Sequenz folgende Nucleotide umfaßt:

26 10 30 50
AGTGCAGAAGAAACCCAAAAGCAACAAACAGGTTTTCGATAAGTACATATATAAGAGG-CCT

30 70 90 110
TTTGTCCCCATCAAAAATGTTACTGTTCTTACGATTGATTACGATTCAAGAAATAGTCA

190 210 230
TTCCCTTCATTTTACTGCAGTTTATTCGGCAGCATCCTCCGCAATTAGCTGCTCCAGTC

40 250 270 290
ACACTACACAGAAGATGAAACGGCACAAATCCGGCTGAAGCTGTCACTCGGTTACTCAG

45

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5 310 330 350
 ATTTAGAAGGGGATTCGATCTGCTGTTGCCATTTCCAAACAGCACAAATAAACGGT
 10 370 390 410
 TATTGTTATAAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTGG
 15 430 450 470
 ATAAAAGAGAGGGCTGAAGCTTCTGTGAGTGAAATACAGCTTATGCCATAACCTGGGAAAC
 20 490 510 530
 ATCTGAACCTCGATGGAGAGAGTAGAAATGGCTGCCATAAGAAGCTGCAGGATGTGCACAAATT
 25 550 570 590
 TGGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTCCAGAGGCCCGAAAAA
 30 610 630 650
 AGGAAAGACAAATGTCTTGGTTGAGAGGCCATGAAAAAGTCTTGGAGAGGCAGACAAAGCTG
 35 670 690 710
 ATGTGAATGTATTAACTAAGCTAAATCCAGTGAAATGAAACAGATATTGTCAGAGT
 40 730 750 770
 TCTGCCTAGAGTCGACTTTGTTCCCACCTGACTTTAGCTCGTACAAATACAAATAC
 45 790 810 830
 TTTTCATTCTCCGTAAACAAACCTGTTCCCATGAAATATCCCTTTCTATTTTCCGTT
 50 850 870
 CGTTACCAACTTACACATACTTATATAGCTA,

40 wobei die Nucleotide 1 bis 173 der Sequenz die MF α 1-Promotorregion und die 5'-nicht-codierende Sequenz ausmachen, es sich bei den Nucleotiden 174 bis 440 um die MF α 1-N-terminal codierende Sequenz handelt, es sich bei den Nucleotiden 441 bis 695 um eine HPTH-Sequenz handelt, es sich bei den Nucleotiden 696 bis 726 um eine HPTH-3'-nicht-codierende Sequenz aus pSSHPTH-10 handelt, das in Fig. 6 gezeigt ist, die Nucleotide 727 bis 732 aus pUC19 stammen, es sich bei den Nucleotiden 733 bis 874 um die MF α 1-3'-nicht-codierende Sequenz und das transkriptionale Terminationssignal handelt.

45

3. Mikroorganismus in Form einer Hefe, vorzugsweise *Saccharomyces cerevisiae*, enthaltend das Plasmid nach Anspruch 1 oder 2.
4. Verfahren zur Herstellung des Plasmids nach Anspruch 1 oder 2, umfassend die Insertion der Nucleotidsequenz von Anspruch 1 oder 2, die für PTH, insbesondere menschliches PTH codiert, in ein Plasmid.
- 55 5. Verfahren nach Anspruch 4, wobei die Insertion durch em uts Klonierung von pSSHPTH-10 durchgeführt wird, dessen Restriktionskart in Fig. 6 g zeigt ist.

Reverendations

5 1. Plasmide pour une insertion dans une levure comportant une séquence nucléotidique codant pour une production et une sécrétion d'hormone parathyroïdienne, plus particulièrement d'une hormone parathyroïdienne humaine, dans lequel la séquence nucléotidique comporte :

5 790 810 830

850 870
10 GATTAACCTTACACACCTTATAGCTT.

15 où M = A ou C; R = A ou G; W = A ou T; S = C ou G; Y = C ou T; H = A ou C ou T; N = A ou G ou C ou T, les nucléotides 1 à 173 de ladite séquence représentant la région du promoteur de MF α 1 et la séquence 5' non codante; les nucléotides 174 à 440 étant la séquence codante N-terminale de MF α 1; les nucléotides 441 à 695 étant une séquence de l'HPTH, les nucléotides 696 à 726 étant une séquence 3' non codante de l'HPTH provenant de pSSHPTH-10 représenté sur la figure 6, les nucléotides 727 à 732 provenant de pUC19, les nucléotides 733 à 874 étant la séquence 3' non codante de MF α 1 et le signal de terminaison de la transcription.

20 2. Plasmide selon la revendication 1, dans lequel la séquence de nucléotides comporte :

10 30 50
AGTGCAGAAGCCAGGTTTGGATAGTACATATATAAGAGGSCCT

70 90 110
TTTGTCCCATCAAAATGTTACTGTCCTACGATTCACTACGATTCAAGAAATAGTC

35 190 210 230
TTCCTTCATTTTACTGAGCTTTATGGAGCATGCTCCCCATGGCTCTCCAGC

40 250 270 290
ACACTACAAACAGATGAAACGGCACAAATTACCGGCTGAAGCTGTCATCGGTTCTG

310 330 350
ATTTAGAAGGGGATTTCGATGTTGCTGTTGCCATTTCCAACAGCACAAATTAACGGGT

370 390 410
TATTCCTTATTAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTAGG

6 430 450 470
 ATAAAAGAGAGCCCTGAGCTCTCTGAGTGAAATACAGCTTATGCATAACCTGGGAAAC

 10 490 510 530
 ATCTGAACTCGATGGAGAGAGTAGAATGGCTGCCAAGAGCTCCAGGATTTCCACATT

 15 550 570 590
 TGTGCCCCCTGGAGCTCCCTAGCTCCCAGAGATGCTGGTCCAGAGGCCCCGAAAG

 20 610 630 650
 AGGAAGACAAATGTCTTGGTTGAGAGCCATGAAAAAGTCTTGGAGAGGCAGACAGCTG

 25 670 690 710
 ATGTGAAATGTATTAACTAAAGCTAAATCCAGTGAAATGAAACAGATATTGTCAGAGT

 30 730 750 770
 TCTGCTCTAGAGTCGACTTTGTTCCCACTGAACTTTAGCTCGTACAAATACAAATAC

 35 790 810 830
 TTTCACTTCTCCGTAAACAAACCTGTTCCCATGAAATACCTTTCTATTTTCTT

 40 850 870
 CGTTACCAACTTACACATACCTATAGCTAT,

35 où les nucléotides 1 à 173 de ladite séquence représentent la région du promoteur de MFα1 et la séquence 5' non codante, les nucléotides 174 à 440 sont la séquence codante N-terminale de MFα1; les nucléotides 441 à 695 sont une séquence de l'HPTH, les nucléotides 696 à 726 sont une séquence 3' non codante de l'HPTH provenant de pSSHPTH-10 représenté sur la figure 6, les nucléotides 727 à 732 proviennent de pUC19, les nucléotides 733 à 874 sont la séquence 3' non codante de MFα1 et le signal de terminaison de la transcription.

40 3. Microorganisme d'une forme de levure, de manière préférentielle *Saccharomyces cerevisiae*, contenant
le plasmide selon la revendication 1 ou 2.

45 4. Procédé pour réaliser le plasmide selon la revendication 1 ou 2, comportant l'insertion de la séquence
nucléotidique selon la revendication 1 ou 2, codant pour la PTH, en particulier la PTH humaine, dans
un plasmide.

5. Procédé selon la revendication 4, dans lequel l'insertion est réalisée en reclonant pSSHPTH-10, dont la
carte de restriction est représentée sur la figure 6.

50

55

10 30 50
ATGATHCCNGCNAARGAYATGGCNAARGTNATGATHGTNATGYTNGCNATHTGYTTYTN

70 90 110
ACNAARWSNGAYGGNAARWSNGTNAARAARMGNWSNGTNWSNGARATHCARYTNATGCAY

130 150 170
AAYYTNGGNAARCAYYTNAAYWSNATGGARMGNGTNGARTGGYTNMGNAARAARYTNCAR

190 210 230
GAYGTNCAYAAYTTYGTNGCNYTNGNGCNCCNYTNGCNCCNMNGAYGCNGGNWSNCAR

250 270 290
MGNCCNMGNAARAARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGGNGAR

310 330
GCNGAYAARGCNGAYGTNAAYGTNYTNACNAARGCNAARWSNCARTRR

M = A or C
R = A or G
W = A or T
S = C or G
Y = C or T
H = A or C or T
N = A or G or C or T.

FIGURE 1

10 30 50
 ATGATGATACTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTGTTT
 70 90 110
 CTTACAAAATCGGATGGAAATCTGTTAAGAAGAGATCTGTGAGTGAATACAGCTTATG
 130 150 170
 CATAACCTGGGAAAACATCTGAACCTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCTG
 190 210 230
 CAGGATGTGCACAATTTGTTGCCCTGGAGCTCCTCTAGCTCCAGAGATGCTGGTTCC
 250 270 290
 CAGAGGCCCGAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAGTCTTGGAA
 310 330
 GAGGCAGACAAAGCTGATGTGAATGTATTAACCTAAAGCTAAATCCAGTGA

FIGURE 2

10 30 50
TATGATGATHCCNGCNAARGAYATGGCNAARGTNATGATHGTNATGYTNGCNAHTGYTT

70 90 110
YYTNACNAARWSNGAYGGNAARWSNGTNAARAARMGNWSNGTNWSNGARATHCARYTNAT

130 150 170
GCAYAAYYTNGGNAARCAYYTNAAAYWSNATGGARMGNGTNGARTGGYTNMGNAARAARYT

190 210 230
NCARGAYGTNCAYAAYTTYGTNGCNYTNGGNGCNCNYTNGCNCCNMNGAYGCNGGNWS

250 270 290
NCARMGNCCNMGNAARAARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGG

310 330 350
NGARGCNGAYAARGCNGAYGTNAAYGTNYTNACNAARGCNAARWSNCARTRRAATGAAA

370 390 410
ACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACTGCTGCTAATTC

430
AAAGCTCTATTA

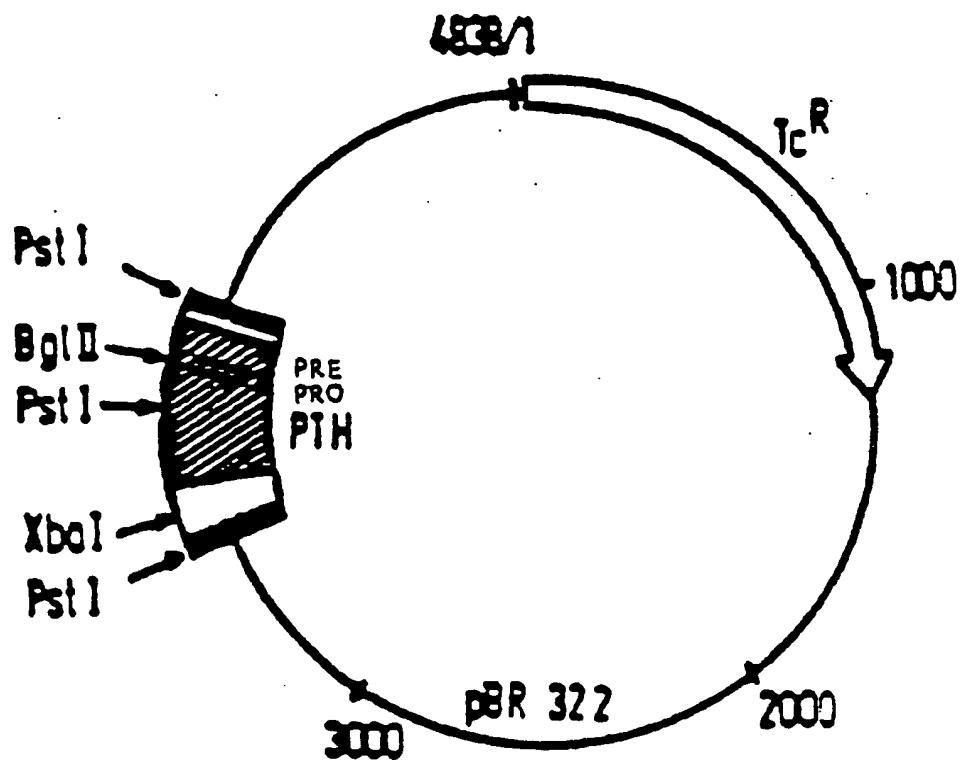
M = A or C
R = A or G
W = A or T
S = C or T
Y = C or T
H = A or C or T
-N = A or G or C or T.

FIGURE 3

10 30 50
TATGATGATACTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTGTTT
70 90 110
TCTTACAAAATCGGATGGAAATCTGTTAAGAAGAGATCTGTGAGTGAAATACAGCTTAT
130 150 170
GCATAACCTGGAAAAACATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCT
190 210 230
GCAGGATGTGCACAATTTGTTGCCCTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTC
250 270 290
CCAGAGGCCCGAAAAAGGAAGACAATGTCTCGTTGAGAGCCATGAAAAAGTCTTGG
310 330 350
AGAGGCAGACAAAGCTGATGTGAATGTATTAACTAAAGCTAAATCCCAGTGAAAATGAAA
370 390 410
ACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCTGCTAATTG
430
AAAGCTCTATTA.

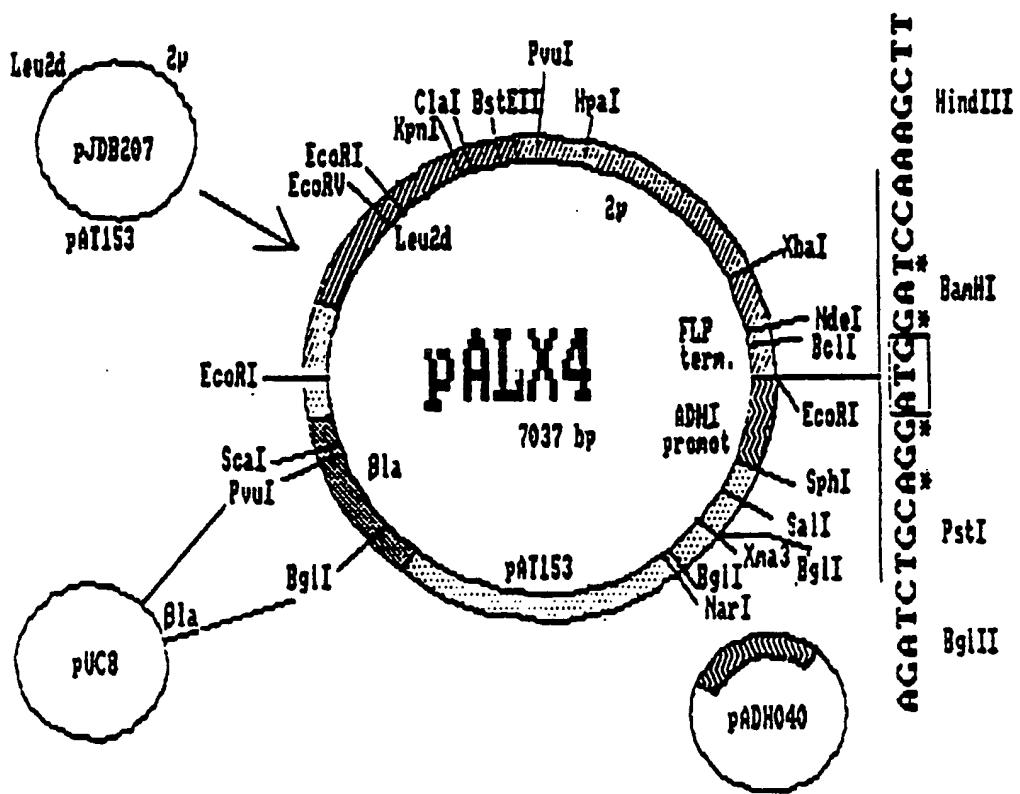
FIGURE 4

FIGURE 5



pSSHPTH-10

FIGURE 6



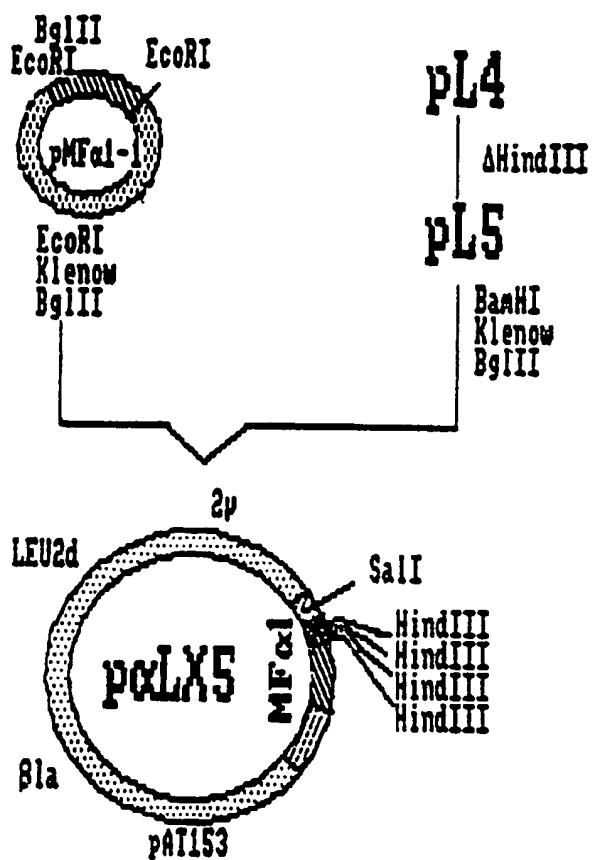


FIGURE 8

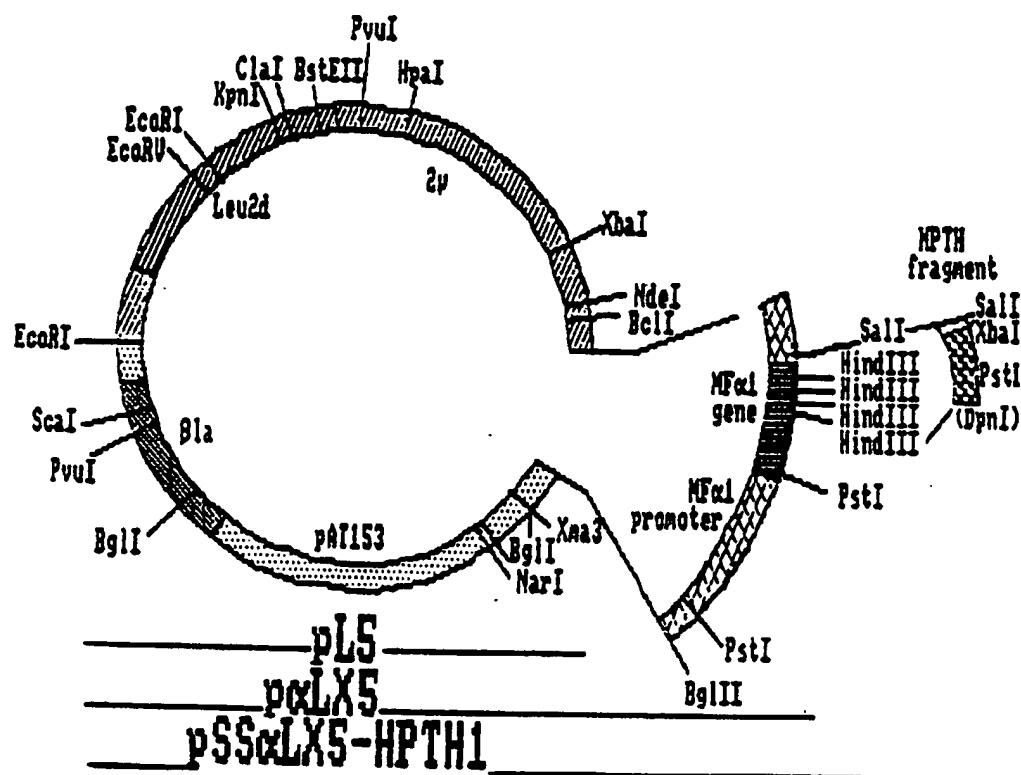


FIGURE 9

10 30 50
AGTGCAAGAAAACCAAAAGCAACAACAGGTTGGATAAGTACATATATAAGAGGGCCT
70 90 110
TTTGTCCCCATCAAAATGTTACTGTTCTTACGATTACGATTCAAGAATAGTTCA
130 150 170
AACAAAGAAGATTACAAACTATCAATTTCATACACAATATAAACGACCAAAAGAATGAGAT
190 210 230
TTCCTTCAATTTTACTGCAGTTTATTGCAGCATTCTCCGCATTAGCTGCTCCAGTC
250 270 290
ACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCACTGGTTACTCAG
310 330 350
ATTTAGAAGGGGATTCGATGTTGCTGTTGCCATTTCAAACAGCACAAATAACGGGT
370 390 410
TATTGTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGTATCTTG
430 450 470
ATAAAAGAGAGGCTGAAGCTWSNGTNWSNGARATHCARYTNATGCAYAAYTNGGNAARC
490 510 530
AYYTNAAYWSNATGGARMGNGTNGARTGGYTNMGNAARAARYTNCARGAYGTNCAYAAYT
550 570 590
TYGTNGCNYTNGGNGCNCCNYTNGCNCCNMNGNAYGCNGGNWSNCARMGNCCNMGNAAARA

FIGURE 10

610 630 650
ARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGGNGARGCNGAYAARGCNG

670 690 710
AYGTNAAYGTNYTNACNAARGCNAARWSNCARTRRAAATGAAAACAGATATTGTCAGAGT

730 750 770
TCTGCTCTAGAGTCGACTTGTCCACTGTACTTTAGCTCGTACAAAATACAATATAC

790 810 830
TTTCATTCCTCCGTAAACAACCTGTTTCCATGTAATATCCTTTCTATTTTCGTTT

850 870
CGTTACCAACTTACACATACTTTATAGCTAT, wherein

M = A or C
R = A or G
W = A or T
S = C or G
Y = C or T
H = A or C or T
N = A or G or C or T

FIGURE 10 (Cont.)

10 30 50
AGTGCAAGAAAACCAAAAAGCAACAAACAGGTTGGATAAGTACATATATAAGAGGCCT

70 90 110
TTTGTCCCATCAAAATGTTACTGTTCTACGATTACGATTCAAGAATAGTTCA

130 150 170
AACAAAGAAGATTACAAACTATCAATTACATACACAATATAAACGACCAAAAGAATGAGAT

190 210 230
TTCCTTCAATTTCAGTTACTGCAGTTTATTGCAGCATCCTCCGCATTAGCTGCTCCAGTCA

250 270 290
ACACTACAAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTACCGTTACTCAG

310 330 350
ATTAGAAGGGGATTCGATGTTGCTGTTGCCATTCCAACAGCACAAATAACGGGT

370 390 410
TATTGTTATAAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTGG

430 450 470
ATAAAAGAGAGGGCTGAAGCTCTGTGAGTGAATACAGCTTATGCATAACCTGGAAAAC

490 510 530
ATCTGAACTCGATGGAGAGAGTGAATGGCTGCGTAAGAAGCTGCAGGATGTGCACAATT

550 570 590
TTGTTGCCCTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTCCAGAGGCCCGAAAAA

FIGURE 11

610 630 650
AGGAAGACAATGTCTGGTTGAGAGCCATGAAAAAGTCTGGAGAGGCAGACAAAGCTG

670 690 710
ATGTGAATGTATTAAC TAAAGCTAAATCCCAGTGA AAAACAGATATTGTCAGAGT

730 750 770
TCTGCTCTAGAGTCGACTTTGTTCCCACTGTACTTTAGCTCGTACAAAATACAATATAC

790 810 830
TTTCATTTCTCCGTAAACAAACCTGTTCCCATGTAATATCCTTCTATTTCGTT

850 870
CGTTACCAACTTACACATACTTTATATAGCTAT

FIGURE 11 (Cont.)

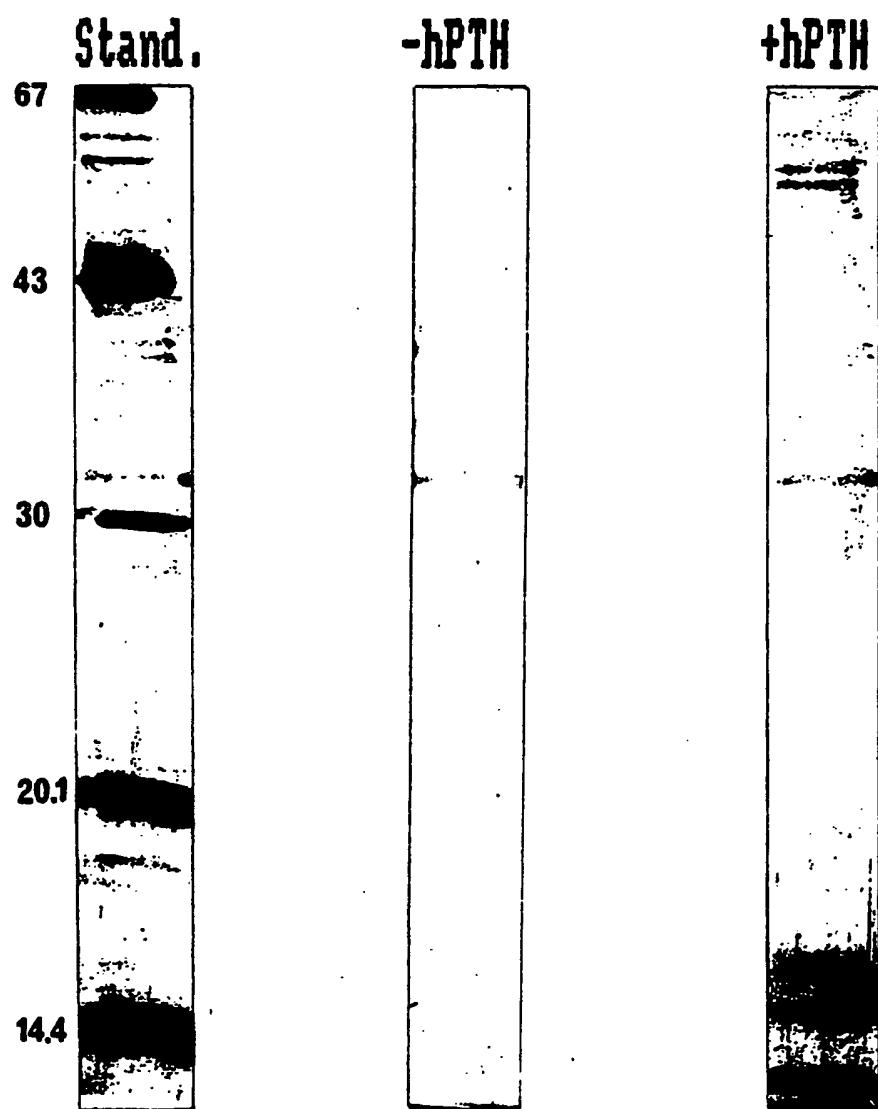


FIG. 12